CD4-Modified Synthetic Peptides Containing Phenylalanine Inhibit HIV-1 Infection In Vitro

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Summary: Phenylalanine-containing peptides from CD4 were synthesized based on chemical similarity with active CD4(81–92)-benzylated peptides. The synthetic peptide FYIFFVEDQKEEDD blocked the binding of gp120 to CD4 and inhibited 50% human immunodeficiency virus (HIV)–induced syncytia formation at a concentration (IC_{50}) of 40–50 μM and HIV p17 expression with an IC_{50} of 67 μM. The peptide is not toxic to cells in vitro. Moreover, acute toxicity studies carried out in Swiss mice showed the peptide to be nontoxic at a dose of 2,000 mg/kg. This phenylalanine-substituted CD4 peptide may prove to be useful in the treatment of AIDS. **Key Words:** Human immunodeficiency virus type 1—CD4-derived peptides—gp 120—Inhibition of human immunodeficiency virus type 1.

The main route of entry of human immunodeficiency virus type 1 (HIV-1) into the host cell has been shown through the interaction of HIV-1 gp120 and CD4 molecules present on the surface of T cells and other cell types (1,2). Several laboratories have prepared recombinant soluble CD4 to test the blocking of HIV-1 gp120 to CD4 and have confirmed that soluble CD4 can efficiently block viral infection in vitro (3–6). These observations led Lifson et al. (7) to test the HIV-1 inhibitory activity of short synthetic peptide fragments encompassing the entire CD4 molecule. They found that benzylated peptides from a region of CD4 (amino acids 76–94) blocked HIV-1 infection whereas nonbenzylated peptides were inactive. Other studies have shown that the benzylated peptide $T_B/YIC_B/E_B/VEDQK_X/EE$ from this region had higher activity than the nonbenzylated peptide (8). Because of the chemical similarity between the benzyl group and the side chain of phenylalanine (Phe), we proposed that the replacement of benzylated amino acids of this peptide by Phe may generate biologically active peptides (9). In the studies reported here, we show that replacement of amino acids by Phe in certain positions of synthetic peptides can generate peptides that inhibit HIV-1 infection in vitro.

METHODS

Peptide Synthesis

Solid-phase peptide synthesis was carried out by the method of Merrifield (10) using the fluorenylmethoxycarbonyl (Fmoc) alternative of Atherton et al. (11). Peptides were purified by high performance liquid chromatography (HPLC) before use.

Cell Cultures

Molt-3 cells were cultured at 37°C and 5% CO_2 in RPMI 1640 (Gibco) supplemented with 10% fetal calf serum (FCS, Flow) and

1% antibiotics (Gibco). The same culture medium was used for HIV-1$_{IIIB}$ chronically infected Molt-3 cells (Molt-3 HIV-1$_{IIIB}$) with the exception of the concentration of FCS (20%), and the addition of 1% anti-PPLO (Gibco).

HT4lacZ-1 cells were cultured in Dulbecco's modification of Eagle medium (DMEM) supplemented with 2 mM $_L$-glutamine, 10% FCS, and 1% penicillin streptomycin.

Titration of HIV-1(III$_B$) Virus

Titration of virus was carried out by means of syncytium formation assay, cocultivating serial dilutions from the viral stocks with uninfected Molt-3 cells or HT4lacZ-1. The concentration of the virus that caused 100–150 syncytia per well was used for the infection inhibition assays.

Syncytia Formation Assay

In the case of Molt-3 syncytium formation assay was carried out as previously described with some modifications (12). Fifty-microliter aliquots of virus were placed in 96-well plates containing either 50 µl solution of peptide in complete medium or medium alone (controls). After incubation at 37°C and 5% CO$_2$ for 1 h, 100 µl of uninfected Molt-3 cells (50,000 cells previously incubated for 30 min in complete medium containing 2 µg ml of polybrene) was added per well. The plates were then incubated for 4–5 days at 37°C and 5% CO$_2$. The syncytia (giant cells greater than three times the diameter of a normal Molt-3 cell) were counted in the entire well with use of an inverted microscope.

The characteristics and use of HT4lacZ-1 cells have been described elsewhere (13). We have adapted their use to perform a quantitative syncytia assay in 96-well plates. Briefly, 10,000 cells (200 µl) well were plated the day before the assay. The following day the medium was removed and 100 µl of peptide solution and 100 µl of diluted virus were added. Controls with no peptide were also made. At the third day postinfection, the medium was removed and the cells fixed for 5 min at room temperature with 200 µl of a phosphate buffered saline (PBS) solution containing 1% formaldehyde and 0.2% glutaraldehyde. After two washes with 0.9% NaCl the cells were incubated during 1 h at 37°C with 200 µl of a reaction mixture containing X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside, Boehringer Mannheim) (400 µg ml), 4 mM potassium ferrocianide, 4 mM potassium ferricianide, and 2 mM MgCl$_2$ in PBS. After two washes, 100 µl of 0.9% NaCl was added per well. The plates were examined under the microscope, and only syncytia with three or more blue nuclei were counted in the entire well.

Fusion Assay

The fusion assay (formation of syncytia from HIV-1-infected and uninfected cells) using Molt-3 cells has been described elsewhere (7). We modified this assay by using uninfected HT4lacZ-1 cells. Thus, 50,000 HT4lacZ-1 cells (100 µl) were added to 5,000 chronically infected Molt-3 HIV-1$_{IIIB}$ cells preincubated for 1 h with 100 µl of serial dilutions of peptide in complete medium. After 24 h, the X-gal staining and counting of syncytia was performed as described for the syncytium formation assay.

HIV-1 p17 Expression

HIV-1 p17 expression was measured on cells fixed in methanol acetone (1:1; vol vol) as described (14). Briefly, the pelleted cells were resuspended in PBS at a concentration of 10^6 per ml. The cells were spotted on toxoplasmosis slides, air dried and fixed in methanol acetone (1:1) for 15 min at room temperature. The slides were next incubated with 10% normal goat serum at room temperature for 30 min and washed four times with PBS. Monoclonal antibody to HIV-1 p17 was added to each well, and the slides incubated for 30 min in a humid chamber at 37°C. The slides were washed with PBS (4 times), incubated with fluorescein isothiocyanate (FITC)-labeled goat anti-mouse immunoglobulin (Ig) G (Cappel Laboratories) for 30 min at 37°C, and subsequently washed with PBS, mounted with 50% (vol vol) glycerol, and examined under a Zeiss Fluorescence microscope. The percentage of cells exhibiting fluorescence in the peptide treated and untreated cultures was compared.

CD4-gp120 Binding Assay

To measure the ability of peptides to block CD4–gp120 interactions, we used the gp120 Capture ELISA (enzyme-linked immunosorbent assay) kit based on plates coated with CD4 protein (American Bio-Technologies). To choose the amount of recombinant gp120 (American Bio-Technologies) to be used in the assay, we measured the binding of gp120 to CD4 by incubation of the plate with 0.2–50 ng of gp120 per well for 1 h at room temperature. Quantitation of the bound gp120 with anti-gp120 antibodies showed that 10 ng of gp120 gave an optical density (OD) of around 1.2, thus, this amount of gp120 per well was used in all subsequent binding inhibition experiments. Basically, gp120 was preincubated for 1 h at room temperature with assay buffer or with different concentrations of peptides and then added to the ELISA plate coated with CD4. The remaining steps of the CD4–gp120 binding assay were performed according to the manufacturer's instructions.

Alternatively, the CD4-coated wells were preincubated with peptide for 1 h at room temperature and washed with assay buffer before adding gp120. The rest of the assay was performed as above.

The inhibition of binding was calculated with use of the following formula:

$$\% \text{ inhibition} = \frac{100 \cdot (OD_{gp120} - OD_{gp120 - peptide})}{OD_{gp120}}$$

Toxicity Studies

Cell Toxicity

Viability of HT4lacZ-1 cells in the presence of peptide was evaluated with use of a modified cell lytic assay described elsewhere (15). It was performed in parallel with the syncytia formation assay.

To 10,000 HT4lacZ-1 cells plated the day before, 200 µl of medium containing different concentrations of the peptides or medium (control wells) are added. At the third day the wells are washed three times with PBS, and cell lysis is detected by staining the plate for 10 min at room temperature, with 20 µl well of a methanol water (1:4 vol vol) solution containing 0.5% crystal

violet. Controls of wells without cells are also made to provide the background caused by staining of the plate (blank wells). Three washes are made by immersion in PBS of the plates, changing the PBS of the container each time. The plates are wiped and 100 µl of 0.1% sodium dodecyl sulfate (SDS) well are added. After complete disaggregation of the cell membranes, the OD at 540 nm is read in a Titertek Multiskan II autoreader and the percentage of viability (%V) is calculated with use of the formula:

$$\%V = \frac{100 \cdot (OD\ peptide\ wells - OD\ blank)}{(OD\ control\ wells - OD\ blank)}$$

Animal Toxicity

Intraperitoneal Acute Toxicity test was carried out on Swiss mice according to the Organization for Economic Cooperation and Development Norms [16] and also to the European Economic Community (EEC) directive [17]. Weight measurements, Irwin test, clinical inspection, and an anatomopathological study were also carried out.

RESULTS

Peptides 1 to 4 from Table 1 were synthesized first. The phenylalanines replacing potentially benzylated amino acids in the sequence of the CD4 peptide TYICEVEDQKEE are in bold. The four peptides were difficult to dissolve in the syncytia inhibition assay medium. Peptide 3 and peptide 4 were solubilized with the help of a sonicator. Because of the low solubility of peptide 1 and peptide 2, these peptides were not tested. The solutions of peptide 3 and peptide 4 were tested for their capacity to inhibit syncytia formation in HIV-1–infected Molt-3 cells. The results showed that peptide 4 was more active than peptide 3 (Table 2).

In a second group of experiments, peptide 5 and peptide 6 were synthesized. Peptide 5 has the same amino acid sequence as peptide 4 but contains two extra aspartic acid residues at the C-terminal end to improve solubility. The inhibition of syncytia formation and the inhibition of p17 synthesis assays show that peptide 6 is more active than

TABLE 1. *Phenylalanine containing CD4 peptides synthesized for the study of inhibition of infection due to human immunodeficiency virus type 1*

Peptides	Amino acid sequence
1	T-Y-I-F-F-V-E-F-Q-K-F-E
2	T-Y-I-F-F-V-E-F-Q-K-F-F
3	T-Y-I-F-E-V-F-D-Q-K-E-E
4	T-Y-I-F-F-V-E-D-Q-K-E-E
5	T-Y-I-F-F-V-E-D-Q-K-E-E-D-D
6	F-Y-I-F-F-V-E-D-Q-K-E-E-D-D

Phenylalanines replacing potentially benzylated amino acids in the sequence of the peptide TYICEVEDQKEE are in bold.

TABLE 2. *Inhibition of infection due to human immunodeficiency virus type 1 by modified CD4 synthetic peptides, with use of Molt-3 cells*

Peptide concentration (µM)	Inhibition			
	Syncytia formation		p17 Synthesis	
	%	IC$_{50}$ (µM)	%	IC$_{50}$ (µM)
TYIFFVEDQKEE (Peptide 3)				
37.5	0		NT	ND
75	0		NT	ND
150	16	300	NT	ND
300	19		NT	ND
TYIFFVEDQKEE (Peptide 4)				
37.5	0		NT	ND
75	0		NT	ND
150	18	262	NT	ND
300	61		NT	ND
TYIFFVEDQKEEDD (Peptide 5)				
28	0		0	
56	0		0	
112	49	116	0	168
168	60		40	
FYIFFVEDQKEEDD (Peptide 6)				
28	0		0	
56	55		45	
112	87	53	70	67
168	91		75	

The phenylalanines replacing benzyl groups are in bold. NT, nontested; ND, not determined; IC$_{50}$, concentration required to inhibit HIV-1–induced syncytia formation or p17 synthesis by 50%.

peptide 5 (Table 2). Moreover, these two peptides have a higher activity than peptide 4 (Table 2). The inhibition of syncytia formation of peptides 5 and 6 was also tested with HT4lacZ-1 cells (Table 3). Clearly, the activities of these peptides are similar to the ones found when using Molt-3 cells. Also, the results of Table 4 show that peptide 6 is able to block the fusion of HIV-1–infected Molt-3 cells to uninfected HT4lacZ-1 cells. As a negative control in these assays we used the peptide TYICEVEDQKEEDD, containing the sequence 81–92 of CD4. This peptide was totally inactive at 300 µM, the highest concentration tested (data not shown).

The ability of peptide 5 and peptide 6 to block the binding of gp120 to CD4 was studied with use of a gp120 capture kit as described previously in the Methods section. Two types of experiments were carried out. In the first experiment, the recombinant gp120 was preincubated with the peptide to be tested and then added to the ELISA plate coated

TABLE 3. *Inhibition of infection due to human immunodeficiency virus type 1 by modified CD4 synthetic peptides with use of HT4lacZ-1 cells*

Peptide concentration (μM)	Inhibition %	IC$_{50}$ (μM)	Cell viability (%)
TYIFFVEDQKEEDD (Peptide 5)			
18.75	1.74		103.98
37.5	9.85		97.95
75	28.11	145	98.74
150	51.53		83.54
FYIFFVEDQKEEDD (Peptide 6)			
18.75	15.83		102.10
37.5	47.10		100.03
75	71	42	95.12
150	96.18		88.50

The phenylalanines replacing benzyl groups are in **bold**. The results are expressed as the average from six wells from two independent experiments. IC$_{50}$ concentration required to inhibit HIV-1-induced syncytia formation by 50%.

with CD4. In the second experiment, the CD4-coated wells were preincubated with peptide, washed with assay buffer to remove unbound peptide, and then incubated with gp120. From Fig. 1A it is clear that peptide 5 and peptide 6, but not the parent CD4-derived peptide TYICEVEDQKEEDD, are able to block the binding of gp120 to CD4. Moreover, peptide 6 is more efficient than peptide 5 in blocking CD4–gp120 interaction, a result in agreement with the higher syncytia inhibition activity of peptide 6. In addition, Fig. 1B shows that preincubation of CD4 with either peptide 5 or peptide 6 does not lead to blockage of the binding of gp120 to CD4.

The toxicity test of peptides 5 and 6 for HT4lacZ-1 cells was carried out in parallel with the syncytia formation assay. These experiments

TABLE 4. *Inhibition of Molt-3 HIV-1$_{IIIB}$ and HT4lacZ-1 fusion by peptide 6 (FYIFFVEDQKEEDD)*

Concentration (μM)	Inhibition %	IC$_{50}$ (μM)
18.75	7.14	250
37.5	11.76	250
75	17.47	250
150	31.92	250
300	59.05	250

The phenylalanines replacing benzyl groups are in **bold**. The results are expressed as the average from six wells from two independent experiments. IC$_{50}$ concentration required to inhibit HIV-1-induced syncytia formation by 50%.

showed that these peptides are nontoxic for these cells (Table 3). Animal toxicity studies of peptide 6 were carried out in Swiss mice. It was observed that with a dose of 2,000 mg/kg, in a single intraperitoneal administration, this peptide provokes neither lethality nor neurobehavioral alterations, neither in the ponderal evolution nor in the general state. In addition to this, anatomopathological alterations were not observed.

In conclusion, in accordance with the results obtained in the Intraperitoneal Acute Toxicity Test for peptide 6, conducted according to OECD guidelines (16), this peptide is nontoxic. A similar conclusion is reached by using guidelines of EEC directive 87/19 (17).

DISCUSSION

The chemical similarity between the benzyl group and the Phe side-chain suggested the possibility that substitution of benzylated amino acids, in benzylated CD4-derived peptides (7), by Phe may provide biologically active compounds (9). Peptide 1 and peptide 2 proved to be very insoluble in the buffer used for the syncytia inhibition assay. This is most likely due to the four and five hydrophobic phenylalanine replacements introduced in the respective original sequences, and also, to the low net negative charge of these peptides under the pH conditions of the assay. Peptide 3 and peptide 4 were more soluble but dissolved with difficulty. To improve the net charge of the Phe-modified peptides, and thus increase their solubility at physiological pH, two extra aspartic residues were added at the C-terminal end. We favored the C-terminal over other regions to avoid disrupting the activity of peptides substituted at the N-terminus. Indeed, peptide T$_B$YIC$_B$E$_B$VEDQK$_{Ac}$EE containing an extra benzyl at the N-terminus (8) is more active than TYIC$_B$E$_B$VEDQKEE (7) suggesting that this benzyl group is important for activity.

Because peptide T$_B$YIC$_B$E$_B$VEDQK$_{Ac}$EE had the higher activity of all reported benzylated peptides (8), we decided to synthesize peptide 6 with two added aspartic acid residues to improve solubility (Table 1). As expected, the two extra aspartic acid residues rendered this peptide soluble despite the presence of the three Phe substitutions. Improvements in solubility and activity were found after adding two aspartic acid residues to peptide 4 to give peptide 5 (Table 2).

The results reported by others (7,8) together with

FIG. 1. Inhibition of gp120 binding to CD4 by synthetic peptides as measured by a gp120 capture enzyme-linked immunosorbent assay. TY**FF**VEDQKEEDD (P5) and F**M**IFFVEDQKEFDD (P6) contain phenylalanine replacements, in bold, in equivalent positions of TYICEVEDQKEEDD used as a control peptide that contains amino acids 81–92 of the wild sequence of CD4. **A:** 10 ng of gp120 was preincubated with peptides before adding these mixtures to CD4-coated wells. **B:** CD4-coated wells were preincubated with peptides prior to the addition of 10 ng of recombinant gp120.
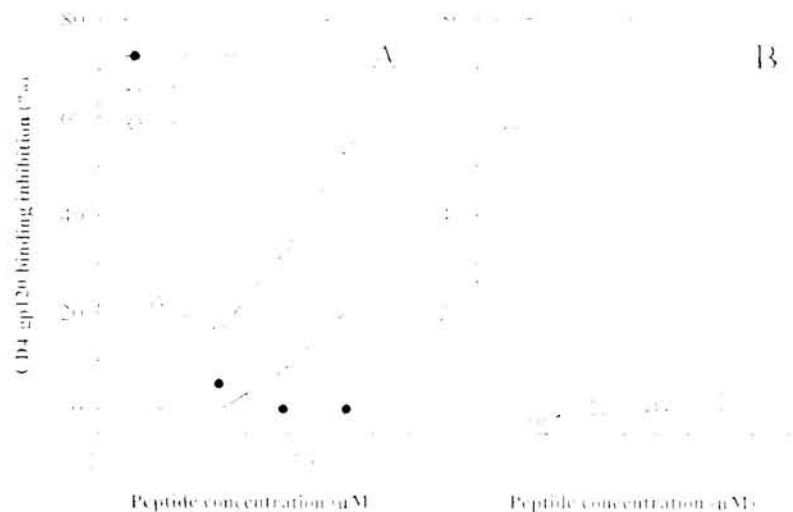
our own suggest that the presence of benzene rings near the N-terminus enhances the biological activity of peptides (peptide TYIC$_{12}$F$_{18}$VEDQKEE is more active than peptide I$_{15}$YIC$_{12}$F$_{18}$VEDQK$_{N}$E, and peptide 6 more than peptide 5). The activity of peptides 5 and 6 were also tested with use of HT4lacZ-1 cells (Table 3). These results confirmed those of Table 2 which show that peptide 6 blocks HIV-1 infectivity in vitro at a lower concentration than peptide 5. Peptide 6 did not show signs of cell toxicity (Table 3) as measured by the viability of HT4lacZ-1 cells incubated with peptide in the absence of HIV-1 under the conditions described in Methods. Moreover, acute toxicity studies carried out in Swiss mice with peptide 6 proved that this peptide is not toxic at a dose as high as 2,000 mg/kg. This result is encouraging in view of the potential use of these peptides in the treatment of AIDS.

Another type of compounds, N-carbomethoxy-carbonyl-prolyl-phenylalanyl benzyl esters (CPEs) (18), has been described as able to block HIV-1 infectivity in vitro (19). These compounds seem to act via interactions with viral membranes because they are active against enveloped viruses but not against nonenveloped viruses (19). They are active in the range of 0.5–1 mM. However, significant toxic effect of these compounds on C8166 cells is detected after 24-h exposure to 1 mM CPEs (19), their toxicity being probably related to alteration of the cell membrane. Benzylated peptides (7,8,20–22) derived from CD4(81–92) are chemically similar to our Phe-substituted peptides, they are noncompetitive inhibitors of CD4 in its binding to gp120 and to a monoclonal antibody to the CDR2-like region of

CD4 (23). As could be expected from the chemical similarity with benzylated peptides, Fig. 1 shows that peptide 5 and peptide 6 were also able to block the interaction between gp120 and CD4. Since this interaction was blocked after preincubation of gp120 with peptides (Fig. 1A) but not after preincubation of CD4 (Fig. 1B), it may be concluded that peptide 5 and peptide 6 bind to gp120 but not to CD4. Also, as shown in Table 4, in the absence of free HIV-1 in the medium, they are able to block the fusion of noninfected Molt-3 cells to HIV-1-infected HT4lacZ-1 cells having gp120 at their surface, a result in agreement with the blocking of the interaction between gp120 and CD4. As opposed to CPEs, peptide 5 and peptide 6 do not show any sign of toxicity, suggesting that their mode of action is different from that of the CPEs.

Peptide TYIC$_{12}$F$_{18}$VEDQKEE, chemically similar to our peptide 4 and the more soluble version, peptide 5, is able to bind gp120 V3-derived peptides (24). Also, this benzylated peptide blocks the binding of soluble rgp120 to anti-V3 antibodies, suggesting that it binds to the third hypervariable region of gp120 (24), which has been described as a domain that is essential for syncytia formation but not for virus binding (25).

The use of short synthetic peptides containing Phe, like peptide 6, may prove to be useful in the treatment of AIDS. Peptide 6 is more active than the CPEs, and has the advantage of being nontoxic in vivo at a dose as high as 2,000 mg/kg (1.1 mmol/kg). Benzylated peptides have been shown to possess a somewhat higher activity than Phe-substituted peptides, although no direct comparison

has been carried out. However, the Phe-substituted peptides are more easily prepared by synthesis and can also be expressed by recombinant technology.

Since the virus level used for the in vitro HIV-1 inhibition assays is probably higher than the circulating virus levels in humans after HIV-1 infection, it is likely that the active dose of peptide 6 necessary to inhibit HIV-1 in vivo may be lower. This would, however, be true only if the peptide is not quickly degraded or cleared from the blood stream.

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